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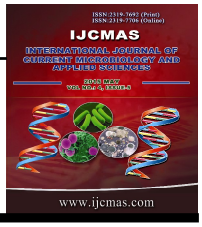

Original Research Article

Evaluation of Synergistic Effect of Kaurenoic Acid Derivatives with Fluconazole against Strains of Fluconazole-Resistant *Candida parapsilosis*

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Keywords

Candida parapsilosis, Resistance, Natural diterpenoids, Synergistic effect, Fluconazole

A B S T R A C T

Candida species are the fourth most common cause of nosocomial bloodstream infections in the United States and the fifth to tenth most common causative pathogen in European studies Although *C. albicans* remains the most common fungal isolate recovered from blood, recent reports indicate a trend towards an increasing prevalence of infections caused by species of *Candida* other than *C. albicans* which are associated with a highly mortality rate. Furthermore, antifungal drug resistance (i.e. resistance to azole compounds) is a prominent feature in the management of invasive mycoses, and its epidemiological characteristics continue to evolve. This scenario leads to seek for new candidates for antifungal drugs able to overcome the resistance issues of *Candida* species. Kaurenoic acid (KA) or ent-kaur-16-en-19-oic acid is a tetracyclic diterpene present in several plants known to exert several pharmacological activities such cytotoxic actions and antimicrobial *in vitro*. The aim of current study was evaluate the potential antifungal of the natural diterpenoids kauren-19-oic acid (KA), 14-hydroxy-kaurane (**1**) and xylopic acid (**2**), and semi-synthetic derivatives of KA (**3-5**) towards *Candida parapsilosis*. Six combinations formed by different diterpenoids kauren-19-oic acid (KA), compounds (1-5), were tested using varied fluconazole concentration. We concluded that the compound 4 (16α-methoxy-(−)-kauran-19-oic methylester) when combined with fluconazole, show activity against strains of *C. parapsilosis* resistant to fluconazole.

Introduction

Candida species are the fourth most common cause of nosocomial bloodstream infections in the United States and the fifth to tenth most common causative pathogen in European studies (Bassetti *et al*., 2007; Picazo *et al*., 2008; Arnold *et al*., 2010). They are the most common cause of invasive fungal infection among hospitalized patients (Zaoutis *et al*., 2005; Ha *et al*., 2012) and are responsible for substantial medical and major economic burdens (Gagne and Goldfarb, 2007). Although *C. albicans* remains the most common fungal isolate recovered from blood, recent reports indicate a trend towards an increasing prevalence of infections caused by species of *Candida* other than *C. albicans* which are associated with a highly mortality rate (Fridkin, 2005; Nucci and Marr, 2005; Sipsas *et al*., 2009; Horn *et al*., 2009).

A principal factor in patients with serious underlying diseases is clinical resistance. Despite of many medical interventions and novel antifungal drugs have been developed, morbidity and mortality rates due to candidemia have hardly improved over the past 20 years (Zaoutis *et al*., 2005; Falagas *et al*., 2006).

Furthermore, antifungal drug resistance (i.e. resistance to azole compounds) is a prominent feature in the management of invasive mycoses, and its epidemiological characteristics continue to evolve (Kanafani and Perfect, 2008). This scenario leads to seek for new candidates for antifungal drugs able to overcome the resistance issues of *Candida* species.

Kaurenoic acid (KA) or ent-kaur-16-en-19-oic acid (Figure 1) is a tetracyclic diterpene present in several plantsknown to exert several pharmacological activities such as anti-inflammatory *in vivo* (Paiva *et al*., 2002; Mizokami *et al*., 2012), smooth muscle relaxant (de Alencar Cunha *et al*., 2003; Tirapelli *et al*., 2005), cytotoxic actions (Costa-Lotufo *et al*., 2002; Cavalcanti *et al*., 2009) and antimicrobial (de Andrade *et al*., 2011; Okoye *et al*., 2012) *in vitro*.

The aim of current study was evaluate the potential antifungal of the natural diterpenoids kauren-19-oic acid (KA), 14-hydroxy-kaurane (**1**) and xylopic acid (**2**), and semi-synthetic derivatives of KA (**3-5**) towards *Candida parapsilosis*.

Materials and Methods

Chemicals: The procedure used for extraction of kaurenoic acid (KA) was described in a previous publication (Cavalcanti *et al*., 2010). The experimental procedures for obtaining 14-hydroxy-kaurane (1), xylopic acid (2), 16α-methoxy-(−)-kauran-19-oic acid (3), 16α-methoxy-(−)-kauran-19-oic methyl ester (4) and 16α-hydroxy-(−)-kauran-19-oic acid (5) were described in detail by Cavalcanti et al. (2009). Their chemical structures are shown in Figure 1.

Isolates: We used four strains of *C. parapsilosis* (Da Silva *et al*., 2011) for these studies that had been isolated from blood samples at the Central Public Health Laboratory (LACEN-CE) and were part of the Collection of Yeasts of the Laboratory of Bioprospection and Experiments in Yeast affiliated with the School of Pharmacy at Federal University of Ceará (LABEL/FF/UFC).

The strains were inoculated on Sabouraud dextrose agar (Himedia Mumbai, India) and incubated at 35°C for 24 h. They were then plated on CHROMagar *Candida* (Himedia Mumbai, India) to assess purity.

Antifungal susceptibility test and evaluation of drug interaction

The broth microdilution (BMD) susceptibility test was performed according to the document M27-A3. Fluconazole (Merck Sharp & Dohme, São Paulo, Brazil) and kaurenoic acid and derivatives (1-5) were dissolved in distilled water and dimethyl sulfoxide (DMSO; Sigma Chemical), respectively. Fluconazole was tested in the range of 0.125–64 μg/mL and kaurenoic acid and derivatives (1-5) in the range of 0.25–128 μg/mL. The strains were classified as susceptible (S) or resistant (R) to fluconazole according to the document M27-S4 (CLSI, 2012). After determining the MIC of each drug, the checkerboard technique was performed.

The percent inhibition of cell growth in the presence of the various drug combinations was determined in relation to the control well containing cells only. Thus, the cells were exposed to varying concentrations (0.25–128 μg/mL) of kaurenoic acid derivatives in combination with 2 μg/mL fluconazole and the interaction between acid kaurenoic and its derivates and fluconazole was determined by calculating the fractional inhibitory concentration index (FICI) as follows: FICI=[FC]/[CFS] + [AKC]/[CAKS], where [FC] and [AKC] represent the MICs of fluconazole and kaurenoic acid and derivatives (1-5) acting in combination, whereas [CFS] and [CAKS] are the MICs of the same drugs acting alone, respectively. The interaction between the drugs was classified as synergistic (FICI < 0.5; SYN), indiferent (0.5 < FICI ≤ 4.0; IND), or antagonic (FICI > 4.0; ANT) (Da Silva *et al.*, 2013, 2014).

Mammalian Cells and cultures

Chinese hamster lung fibroblasts (V79 cells) were kindly provided by Dr. J.A.P. Henriques (Federal University of Rio Grande do Sul, Porto Alegre, Brazil). V79 cells were cultivated under standard conditions in MEM with Earle's salts.

All culture media were supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 μg/mL penicillin, and 100 μg/mL streptomycin at 37°C with 5% CO_2. For evaluation of cytotoxic effects, cells were grown for 2 days prior to treatment with the test substances, and afterwards, the medium was replaced with fresh medium containing the test substance or DMSO solution for control. The final concentration of DMSO in the culture medium was kept constant, less than 0.1% (v/v) (Cavalcanti *et al.*, 2009).

Inhibition of mammalian V79 cell proliferation – MTT test

Cell growth was quantified by the ability of living cells to reduce the yellow dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetra zolium bromide) (MTT, Sigma Chemical) to a purple formazan product. For the experiments, V79 cells were plated in 96-well plates (0.3 x 106 cells/well), and test compounds (0.156 to 100 μg/mL), dissolved in DMSO (0.1%), were then added to each well, followed by incubation for 24 h.

Afterwards, the plates were centrifuged and the medium replaced by fresh medium (150 μL) containing 0.5 mg/mL MTT. Three hours later, the MTT formazan product was dissolved in 150 μL DMSO and absorbance was measured using a multiplate reader (Spectra Count, Packard, Ontario, Canada).

The effect of the test substances was quantified as the percentage of control absorbance of the reduced dye at 595 nm. Experiments were carried out in duplicate and repeated at least three times (Cavalcanti *et al.*, 2009).

Alkaline comet assay

Cultured V79 fibroblasts were plated at a concentration of 0.6 x 10^6 cells/mL and incubated for 6 h with tested compounds (100 µg/mL). MMS (4 x 10-5 M) was used as a positive control. The alkaline version of the comet assay (single cell gel electrophoresis) was performed as described by Singh *et al*. (1988) with minor modifications (Hartmann and Speit, 1997). Slides were prepared in duplicate, and 100 cells were screened per sample (50 cells from each duplicate slide), using a fluorescence microscope (Zeiss) equipped with a 515–560 nm excitation filter, a 590 nm barrier filter, and a 40x objective. Cell scoring and the calculation of damage index were performed according to the protocol described above (yeast alkaline comet assay) (Mioreli *et al*., 2008).

Cytokinesis-block micronucleus assay

Cultured V79 fibroblasts were plated at a concentration of 0.6 x 10^6 cells/mL and incubated for 6 h with tested compounds (100 µg/mL). MMS (4 x 10-5 M) was used as a positive control. After treatment, the cultures were washed twice with medium and cytochalasin B (3 µg/mL) was added to the cultures at 44h post-initiation, as described by Fenech (2000).

Cells were harvested 72h after the start of treatment, resuspended in a 75mM KCl solution, maintained at 4◦C for 3min (mild hypotonic treatment), and fixed with cold methanol/acetic acid (3:1) solution. This fixation step was repeated twice, and finally, cells were resuspended in a small volume of methanol/acetic acid (3:1) solution and dropped onto clean slides.

Slides were stained with 10% Giemsa (pH 6.8) for 4 min, mounted and coded prior to microscopic analysis. Micronuclei were counted in 2000 binucleated cells with well-preserved cytoplasm. The identification of micronuclei was carried out according to Fenech (2000).

Result and Discussion

Synergistic effect of kaurenoic acid derivatives and fluconazole

The fluconazole susceptibility profiles of the *C. parapsilosis* strains were assessed using the microdilution technique previously described (CLSI, 2012). Table 1 showed no variation in the susceptibility of different strains tested with fluconazole. All strains studied showed MIC 50 values above 64 µg/mL. The synergism between kaurenoic acid derivatives and fluconazole was determined using the checkerboard technique, whose association of compound 4 with fluconazole showed synergistic effect on fluconazole-resistant strains (FICI $\leq$ 0.50).

Cytotoxic Activity of kaurenoic acid and derivatives in V79 cell

Table 2 showed that kaurenoic acid and derivatives (1-5) showed moderate cytotoxicity against human leukocytes as analyzed by the MTT assay compared with the control group ($p < 0.05$). The compounds 1-5 showed no cytotoxicity when treated alone compared to the control.

Genotoxicity effect in V79 cell

In order to evaluate the kaurenoic acid and derivatives (1-5) genotoxicity, we investigated whether this compound could induce DNA damage applying the *in vitro* alkaline comet test. This test is the most frequently used assay for routine screening of potential genotoxic agents (Mioreli *et al*., 2008) and can be performed with a variety

of cell types, including V79 cell lines. As shown in Figure 2, the compounds 1-5 did not generate significant DNA damage in comparison to untreated cells ($p < 0.001$). However, the kaurenoic acid induced a significant ($p < 0.001$) increase in DNA damage in V79 cell.

Mutagenic effect in V79 cell

Results of mutagenicity tests are shown in Figure 3. The compounds 1-5 were neither cytotoxic nor mutagenic, at the concentration range employed in V79 fibroblasts, since the survival rate did not decrease. However, the kaurenoic acid showed mutagenic against V79 cell.

The fluconazole susceptibility profiles of the *C. parapsilosis* strains were assessed using the microdilution technique previously described (CLSI, 2012). Table 1 shows no variation in the susceptibility of different strains tested with fluconazole. All strains studied showed MIC50 values above 8 μg/mL. The antifungal resistance, especially to azoles, has emerged as a major clinical problem for immunocompromised patients and patients with high-risk hospitalized fungal infections (Pfaller, 2012).

Because this context, much effort has been and is being done to solve this problem, such as improving the effectiveness of using antifungal combination therapy and the search for new molecules with antifungal activity (GUO *et al*, 2008; Da Silva *et al*, 2013; Da Silva *et al*, 2014) published data on the antifungal activity of the respective compounds against species of *Candida parapsilosis* resistant to fluconazole demonstrated different activities between the molecules, which can remote in to a structure-activity relationship.

The synergism between acid kaurenoic and its derivate and fluconazole was determined using the checkerboard technique, whose association of compound 4 with fluconazole showed synergistic effect on fluconazole-resistant strains ($FICI \leq 0.50$).

In a paper published by Zore *et al*. (2011), terpenes 6 were tested for their potential effect anticandida, showing that these molecules may not only be used as antifungal agents but also as synergistic agents with conventional drugs such as fluconazole. Thus a possible use of these compounds in combination with antifungal agents fluconazole in the case, can promote better efficacy of the drug by administration of lower doses. In addition, the combination therapy may be used in an attempt to prevent or delay the appearance of resistant populations in vivo pathogenic fungi (Estrella, 2004).

The diterpenes kaurenoics emerge as potential molecules, bearing in mind that several studies have demonstrated diverse biological effects such as bacterial activity and tumor cytotoxicity (Kubo *et al.*, 2004; Kondoh *et al.*, 2004; Cavalcanti *et al.*, 2010). In the present study, the cytotoxic, genotoxic and mutagenic activity of the five kaurenoic acid derivatives (1–5) was also evaluated through the MTT assay, comet and micronucleus V79.

As shown in Table 2, neither the derivatives (1–5) was the MTT cytotoxicity (IC50 > 25 ug / mL). Already kaurenoic acid (KA) had IC50 of 7.43 (7.25–7.91). As shown in Figure 2, the five compounds derived from kaurenoic acid (KA) tested did not cause DNA damage in V79 cells compared to the control group ($p < 0.05$). Treatment of cells with DNA damage caused KA.

Table.1 Synergistic effect of fluconazole with kaurenoic acid (KA) and derivatives (15) against strains of *Candida parapsilosis* resistant to fluconazole and isolated in Ceará

		MIC [b]																		
Strains[a]	**FLC[b] (µg/mL) MIC_{50} 24 h**	**Standard MIC**						**Combination MIC [d] (2ug/mL)**												**FICI[e]**
		KA	**1**	**2**	**3**	**4**	**5**	**KA**	**FLC**	**1**	**2**	**3**	**4**	**5**	**KA**	**1**	**2**	**3**	**4**	**5**	**INT[f]**
C. parapsilosis 1	≥ 8	>128	>128	>128	>128	>128	>128	>128	2	>128	>128	>128	<0.25	>128	17	17	17	17	0.033	17	**A/A/A/A/S/A**
C. parapsilosis 2	≥ 8	>128	>128	>128	>128	>128	>128	>128	2	>128	>128	>128	<0.25	>128	17	17	17	17	0.033	17	**A/A/A/A/S/A**
C. parapsilosis 3	≥ 8	>128	>128	>128	>128	>128	>128	>128	2	>128	>128	>128	<0.25	>128	9	9	9	9	0.018	9	**A/A/A/A/S/A**
C. parapsilosis 4	≥ 8	>128	>128	>128	>128	>128	>128	>128	2	>128	>128	>128	<0.25	>128	17	17	17	17	0.033	17	**A/A/A/A/S/A**

aFLC-resistant strains of *Candida parapsilosis* isolated from biological samples.

bFLC – Fluconazole. AKC- kaurenoic acid and derivatives (1-5). The MIC was defined as the lowest concentration that produced a 50% reduction in growth of fungal cells after 48h of incubation. The procedure was performed according to CLSI protocol M27-A3. Values are expressed in µg/mL for FLC and KA and derivatives (1-5). MICs represent geometric means of at least three MICs determined on different daysc The synergistic effect of FLC and FLAV was calculated based on FICI (fractional inhibitory concentration index FICI=[FC]/[CFS] + [AKC]/[CAKS], where [FC] and [AKC] represent the MICs of fluconazole and kaurenoic acid and derivatives acting in combination, whereas [CFS] and [CAKS] are the concentrations of the same drugs acting alone. The interpretation was performed according to the value of FICI < 0.5 = synergism (SYN); 0.5 < FICI ≤ 4.0 = indifference (IND); and FICI > 4.0 = antagonism (ANT).

Table.2 Cytotoxic activity of kaurenoic acid (KA) isolated from Xylopia sericeae and compounds (1-5) on Cells V79. Data are presented as IC50 values and 95% confidence interval (CI 95%) from three independent experiments, performed in triplicate

	CI 95% (µg/mL)					
	KA	**1**	**2**	**3**	**4**	**5**
Cells V79	7,43 (7.25-7.9)	>100	>100	>100	>100	>100

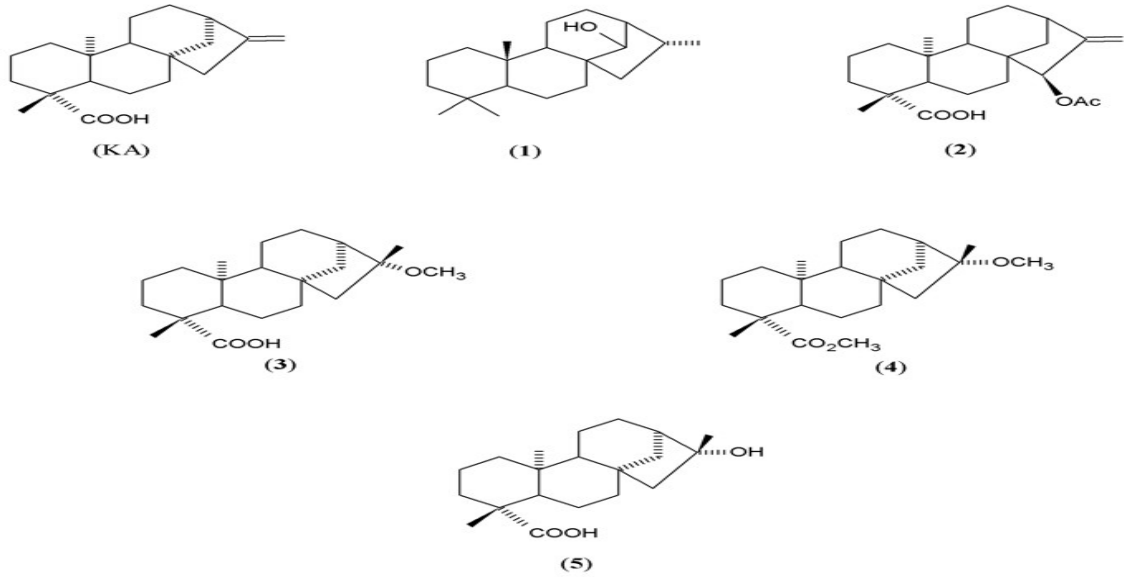

Figure 1, Chemical structures of the kaurenoic acid(KA) and derivatives (1-5) used in the present study.

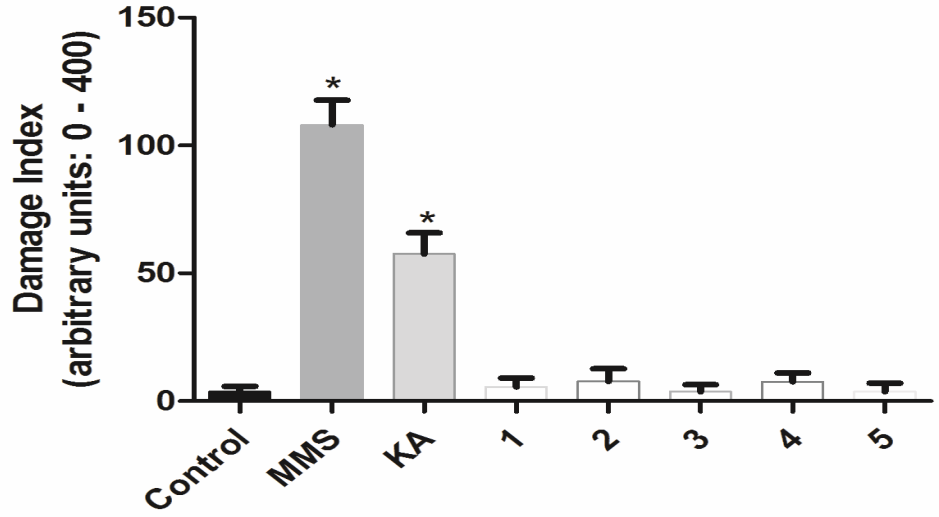

Figure 2. Effect of kaurenoic acid (KA) isolated from *Xylopia sericeae* and compounds (1-5) on the damage index in human leukocytes, tested in the alkaline comet assay after 24h of treatment. Bars represent the mean ± S.E.M. of three independent experiments. *p < 0.001 vs control (ANOVA, Tukey's test). DMSO (0.1%) and MMS (4×10−5 M) were used as the negative and positive controls, respectively.

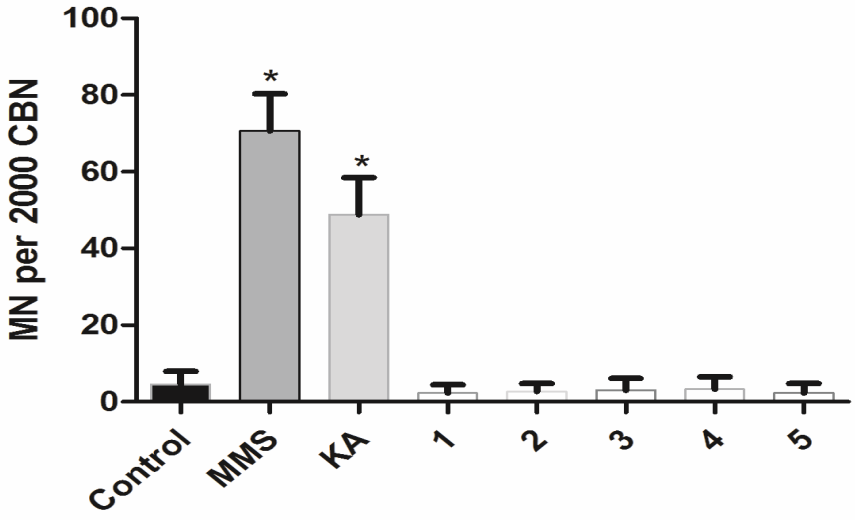

Figure 3. Effect of kaurenoic acid (KA) isolated from *Xylopia sericeae* and compounds (1-5) inthe *in vitro* micronucleus assay after a 24-h treatment of human leukocytes. Bars represent the mean ± S.E.Mof three independent experiments. *p < 0.001 vs control (ANOVA, Tukey's test). DMSO(0.1%) andMMS (4×10−5 M) were used as the negative and positive controls, respectively. Micronuclei (MN) were counted in 2000 binucleated cells (BNC) scored with well-preserved cytoplasm.

Previous work with the compounds (1-5) determined that its cytotoxic effect in part be due to a partial inhibitory effect of topoisomerase (topo I) (Cavalcanti *et al*., 2009). However in a study by Cavalcanti *et al*. (2010) demonstrated that the compounds (1-5) merges mainly with DNA, can induce both apoptosis and necrosis in cultured HL - 60 cells.

The mutagenic potential of compounds (1-5) was also evaluated by testing micronuclei induction with the use of cytochalasin B (cytokinesis blocker). After 24 hours exposure, it was observed that the derivatives of KA (1-5) had low activity against binucleated cells from peripheral blood (BNC) (Figure 3). However BNC treated with KA shown to be most sensitive. According to Cavalcanti *et al*. (2009), KA is genotoxic in vitro and in vivo and mutagenic in yeast cells, probably due to inhibition of topoisomerase I.

In summary, the present data suggest that these compounds may be used as antifungal agents for the treatment of candidemia. The present study indicates a synergistic activity of the compound 16α -hydroxy- (-) - kauran - 19 - oic acid against strains of fluconazole-resistant *C.parapsilosis*. The respective compound showed no genotoxic activity nor mutagenic potential which gives it an advantage, as the search for compounds with low toxicity anticandida activity is always current and relevant topic (Rajeshkumar and Sundararaman, 2011; Tobudic *et al*., 2012). However, other studies focused on the structural modification of compounds (1-5) as well as the mechanisms of action in strains of *Candida* spp. are currently in progress.

Conclusion

In conclusion, the natural diterpenoids kauren-19-oic acid (Compound 4), presented a synergistic effect with fluconazole *in vitro* against strains of fluconazole-resistant *Candida parapsilosis*. In summary, the results suggest that the compound 16α-hydroxy-(-)-kauran-19-oic acid can be used as an adjuvant in combination with antifungals for the treatment of candidemias, although a study with a larger number of strains is necessary to establish this conclusion.

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